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Divergent Response Profile in Activated Cord Blood T cells from First-born Child Implies Birth-order-associated *in Utero* Immune Programming

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management, and interpretation of the data; or preparation, review, or approval of the manuscript.

ABSTRACT

Background: First-born children are at higher risk for development of a range of immune-mediated diseases. The underlying mechanism of 'birth-order-effects' on disease risk is largely unknown, but *in utero* programming of the child's immune system may play a role.

Objective: We studied the association between birth-order and the functional response of stimulated cord blood T cells.

Method: Purified cord blood T cells were polyclonally activated with anti-CD3/CD28-coated beads in a subgroup of 28 children enrolled in the $COPSAC_{2010}$ birth cohort. Expression levels of seven activation markers on helper and cytotoxic T cells as well as the percentage of $CD4^+CD25^+$ T cells were assessed by flow cytometry. Production of IFN-γ, TNF-α, IL-17, IL-4, IL-5, IL-13 and IL-10 was measured in supernatants.

Results: IL-10 secretion (P = 0.007) and CD25 expression on $CD4^+$ helper T cells (P = 0.0003) in activated cord blood T cells were selectively reduced in first-born children, while the percentage of $CD4^+CD25^+$ cord blood T cells was independent of birth-order.

Conclusion: First-born infants display a reduced anti-inflammatory profile in T cells at birth. This possible *in utero* 'birth-order' T cell programing may contribute to later development of immune-mediated diseases by increasing overall immune reactivity in first-born children as compared to younger siblings.

Clinical Implications or Key Messages

The prenatal period of life is a developmental window in which the fetal T cell immune response seems to be programmed by maternal parity status.

Capsule Summary

The authors report a reduced anti-inflammatory bias in the T cell function of first-born child. This *in utero* programming of immunosuppressive mechanisms could contribute to birth-order-associated reductions in immune-mediated disease prevalence.

Key words

Parity number, neonates, T cells, immune regulation, *in utero* programming

Contributions

The guarantor of the study is HB who has been responsible for the integrity of the work as a whole, from conception and design to acquisition of data, analysis and writing of the manuscript. MK contributed to acquisition of data, performed classical statistical analysis as well as multivariate data analysis and wrote the manuscript. JML, AHT and SB contributed to conception, design and acquisition of data and writing of the manuscript. HMW contributed to acquisition of data. MAR contributed to data analysis. All co-authors have contributed to the analysis and interpretation of the data, and have provided important intellectual input and approval of the final version of the manuscript. **Competing interests**: The authors have no conflict of interest.

Abbreviations

APC	Allophycocyanin
$COPSAC_{2010}$	Copenhagen Prospective Studies on Asthma in Childhood
CD:	Cluster of differentiation
CD40L:	CD 40 ligand (CD154)
CTL:	Cytotoxic T lymphocyte
FITC	Fluorescein isothiocyanate
GLM	General linear models
ICOS:	Inducible T cell co-stimulator (CD278)
IFN-γ:	Interferon-γ
IL:	Interleukin
MFI:	Mean fluorescence intensity
PC:	Principal component
PCA:	Principal component analysis
PE:	Phycoerythrin
PC7:	R-phycoerythrin-cyanine 7
PE-Cy7:	PE-Cyanin 7
PHA:	Phytohaemagglutinin
Th:	T helper
TNF-α:	Tumor necrosis factor-α
Tregs:	Regulatory T cells
Tr1:	Type 1 regulatory T cells

INTRODUCTION

A 'birth-order-effect' on disease risk has been known for several decades (1) relating to a higher risk in the first-born child for a number of diseases including lymphoma (2), testicular cancer (3,4), type 1 diabetes mellitus (5), eczema, rhinitis and asthma (6–8). Since these diseases are all influenced by dysfunctional immune activation, we hypothesized that the birth-order may affect early immune function and be programmed already during pregnancy.

Throughout pregnancy the mother's immune system is challenged by the hemi-allogeneic fetus and in order to accomplish a successful pregnancy her immune system is dynamically modulated from conception to delivery (9). Essential for implantation and maintenance of pregnancy is several hormones, including estrogen, which presumably promotes expansion of regulatory T cells (Tregs) and production of anti-inflammatory interleukin-10 (IL-10) within the mother as well as at the maternal-fetal interface (10–14). It is also reported that long-lasting fetus-specific immune cells develop in maternal circulation during pregnancy (15,16) and rapidly expand in secondary pregnancies with the same father (17). An increased number of Tregs specific for paternal antigens is found in secondary pregnancies (18). All-together this suggests that an increased immune suppression occurs in multiparous women. In relation to the general maternal-fetal interplay, it is reported that anti-inflammatory, but not pro-inflammatory, mediator levels correlate between the pregnant mother and her fetus (19), as does estrogen levels (20). These findings support a bidirectional immunological reflection of an anti-inflammatory milieu between mother and child. It is, however, unknown if changes in this maternal-fetal tolerance of newborns with increased birth numbers are a result of *in utero* programming of immune function.

We aimed to study if T cell function are differentially encoded in newborns of primiparous (first pregnancy) and multiparous (second or more pregnancies) mothers. We purified T cells

from cord blood and stimulated these with anti-CD3/CD28-coated beads to induce polyclonal stimulation. T cell function was assessed by measurement of seven activation markers on $CD4^+$ helper and $CD8^+$ cytotoxic T cells, respectively. Secreted cytokines characteristic of T helper 1 (Th1) cell (interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α)), Th2 cell (IL-4, IL-5, IL-13), Th 17 cell (IL-17) and Tregs (IL-10) immunity were measured in supernatants. Moreover, we characterized the percentage of $CD4^+CD25^+$ T cells within unstimulated cord blood T cells. These parameters of T cell function were studied in relation to maternal parity to examine if *in utero* programming of the T cell compartment is influenced by birth-order.

METHODS

Study population

A total of 28 full-term, healthy neonates born from September 2009 to March 2010 were enrolled in this study. The neonates were part of the ongoing unselected Copenhagen Prospective Study on Asthma in Childhood 2010 birth-cohort ($COPSAC_{2010}$) of 700 children recruited in Zealand, Denmark, during 2009-2010 (21). Parental atopy was defined from a history of doctor-diagnosed asthma, rhinitis, and/or atopic dermatitis. This was determined by structured clinical interviews performed by the research doctors in gestation week 24. The study was conducted in accordance with the guiding principles of the Declaration of Helsinki and approved by the Ethics Committee for Copenhagen (H-B-2008-093) and the Danish Data Protection Agency (j.nr. 2008-41-2599). Both parents gave their informed consent before enrollment of the child.

Cord blood sampling and shipment

Cord blood was collected postpartum by needle puncture of the umbilical vein. The blood was stored in a citrate phosphate dextrose-containing bag (catalog 791-01U, Pall, NY) and immediately transported to the laboratory by courier and processed within 24 hours after birth.

T cell purification and activation

Cord blood $CD3^+$ T cells were isolated using the Dynabeads® FlowComp™ Human CD3 kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. In brief, T cells were purified from 40 x 10^6 total nucleated cord blood cells by positive selection using anti-CD3 antibody and magnetic beads. To release $CD3^+$ cells from beads, cells were incubated with FlowComp™ Release Buffer. For all individuals, the purity of $CD3^+$ T cells was

142 determined to be greater than 98% by flow cytometry (anti-CD3/APC-eFlour780 antibody
143 (eBioscience, San Diego, CA)).

144 Isolated T cells were cultured (10^6 cells/mL, 200 ul in 96 U-bottomed plates) and activated
145 using Dynabeads® Human T-Expander CD3/CD28 (Invitrogen) in complete medium (RPMI
146 1640 containing 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, 100 U/mL
147 penicillin, 100 ug/mL streptomycin (all from Lonza)). Control conditions were unstimulated
148 T cells. Cultures were kept in a humidified 37°C, 5% CO_2 incubator for 24h.

149 **T cell activation markers**

150 T cell surface expression of activation markers CD25, CD27, CD30, CD69, CD71, CD154
151 (CD40L) and CD278 (ICOS) was determined on $CD3^+CD4^+$ helper and $CD3^+CD8^+$
152 cytotoxic T cells following activation. 4 x 10^5 T cells were stained in FACS buffer (PBS,
153 0.1% sodium azide, 1 % heat-inactivated fetal bovine serum for 30 min at 4˚C using the
154 following antibodies: Fluorescein isothiocyanate (FITC)-conjugated anti-hCD71, R-
155 Phycoerythrin (PE)-conjugated anti-hCD69, PE-anti-hCD30, PE-Cyanin 7 (PE-Cy7)-
156 conjugated anti-hCD278, allophycocyanin eFluor 780 (APC-eFlour780)-anti-hCD3, eFlour
157 450-conjuated anti-hCD4 (all from eBioscience), R-Cy7-anti-hCD25 (Beckman Coulter,
158 Fullerton, CA), Amcyan-conjugated anti-hCD8 (BD Biosciences, Franklin Lakes, NJ),
159 AlexaFlour647 anti-hCD27 and AlexaFlour488 anti-hCD154 (BioLegend, San Diego, CA).
160 Cells were analyzed on a BD FACSCanto™ II (BD Biosciences) followed by data analysis
161 in FlowJo v.7.6.5 (Tree Star, Ashland, OR). The surface marker expression levels were
162 determined as the mean fluorescence intensity (MFI) in both stimulated and unstimulated T
163 cells. The percentages of $CD4^+CD25^+$ and $CD8^+CD25^+$ T cells were determined in
164 unstimulated samples. Representative histograms of surface marker expressions before and
165 after activation can be found in Supplementary Figure S1.

T cell effector cytokine production

Supernatants were collected 24h after initiation of poly-clonal T cell activation and stored at -80˚C until analysis. The level of IFN-γ, TNF-α, IL-4, IL-5, IL-10, IL-13 and IL-17A (IL-17) were measured in supernatants using a custom Meso Scale Discovery Multi-plex human cytokine assay (Meso Scale Discovery, Gaithersburg, MD). Assays were performed according to manufacturer's recommendations, but included sample incubation over night at 4°C on shaker and the use of an eight-point standard curve (0.65 - 10,000 pg/mL) for increased sensitivity and dynamic range. Samples were read using the Sector Imager 6000 (Meso Scale Discovery). Detection limits were measured in pg/mL, calculated as mean of blanks + 3x standard deviation and were as follows: IFN-γ: 0.93, TNF-α: 0.66, IL-4: 0.88, IL-5: 0.65, IL-10: 0.76, IL-13: 4.1 and IL-17: 1.7. Signals from unstimulated T cells were below the detection limit. Signals below the lower detection limit were set to 0.5 x the lower detection limit and samples with zero values were set to 0.1 x the lower detection limit. For IL-4, 21% of the measurements were below the detection level, and 11% were undetectable; for IL-5, 18% of the measurements were below the detection level; for IL-17, 11% of the measurements were below the detection level and 25% were undetectable.

Statistical analyses of immune mediator measurements

Data was analyzed by conventional statistics and explored using principal components analysis (PCA). If needed, effector mediator levels were log-transformed before analysis to obtain normality distribution. Differences in immune mediator levels between groups by sex, maternal parity and parental atopy were tested by unpaired Student's t-test. General linear models (GLM) were applied with immune mediator levels as dependent variables and sex, parity and parental atopy as independent covariates (Supplementary Table S1). Estimates of

scaled, adjusted dependent variables (z-scores) were expressed as differences between groups with corresponding 95% confidence intervals.

PCA was applied to all 21 variables of T cell activation markers and cytokines after scaling. The first principal component (PC1) in the PCA accounts for the largest variation in the dataset; the second principal component (PC2) accounts for the second largest variation in data, and so forth. Projections of data onto PCs reveal new underlying latent variables (the scores). By use of a PCA algorithm, we obtained the PCs describing the systematic variation in data across the 21 variables; hence revealing the biological profiles that dominate the dataset. We applied the scores as dependent variables in the uni- and multivariate statistical analysis.

Statistical significance was defined by $P < 0.05$. We used Bonferroni correction separately on the two measured compartments: cytokines and surface markers. The PCA further surpassed multiple testing issues by reducing the number of variables to a few latent factors. Data processing was performed with R v. 2.15.3 software (http://www.R-project.org, R Development Core Team, Vienna, Austria). PCA results were illustrated by use of the R add-on Ade4 package (22).

RESULTS

Study population characteristics

The study population is part of the $COPSAC_{2010}$ cohort and included 28 full-term healthy neonates born in the period from September to March. Baseline characteristics of the neonates enrolled in the study cohort did not differ significantly from neonates of the full cohort with respect to factors that may confound the present study: sex, primiparity, maternal atopy and paternal atopy (Table 1).

Birth-order influences CD25 expression and IL-10 levels in activated cord blood T cells

Activated cord blood T cells from first-born infants were found to produce lower IL-10 levels ($P = 0.007$) and display reduced CD25 expression on $CD4^+$ helper T cells ($P = 0.0003$) as compared to those of later-born infants (Figure 1). None of the other T cell cytokines or activation markers differed by birth-order (Figure 1). IL-10 production and CD25 expression on $CD4^+$ helper T cells were also found to be significantly different in first-born infants compared to those of later-born after adjusting for sex and parental atopy (Table 2 and 3). Sex and parental atopy was not associated with cytokine production or activation marker expression levels in activated cord blood T cells (Table 2 and 3).

The percentage of $CD4^+$ helper and $CD8^+$ cytotoxic T cells within the pool of cord blood $CD3^+$ T cells were unaffected by primiparity as well as sex and parental atopy (Table 3). The general activation characteristics and correlations between the twenty-one T cell functional parameters of activated cord blood T cells are displayed in Supplementary Figure S2-S4.

We found that IL-10 levels positively correlated with the expression of CD25 on $CD4^+$ helper T cells in activated cord blood T cells (Figure 2, $P = 0.006$). Consistent with the

univariate analysis in Figure 1; first-born infants displayed lower IL-10 and CD25 expression compared to later-born infants (Figure 2).

We then tested if increased IL-10 production and CD25 expression on $CD4^+$ T cells was associated with enhanced numbers of resting $CD4^+CD25^+$ in cord blood that may represent Treg. An apparent $CD4^+CD25^+$ population was identifiable in unstimulated cord blood T cells (Figure 3A). As CD25 can also be a marker for recent activation, we also analyzed for CD25 expression levels on unstimulated $CD8^+$ T cells that would be apparent if cord blood T cells were activated *in vivo*. No CD25 expression was found on unstimulated $CD8^+$ T cells (Figure 3B), suggesting that the cord blood $CD4^+CD25^+$ cells represent a Treg subset, and not readily activated T cells present within cord blood. When comparing the percentage of circulating cord blood $CD4^+CD25^+$ cells in first-born and later-born infants, we found no differences (Table 3). However, the percentage of cord blood $CD4^+CD25^+$ cells was found to correlate positively with the levels of secreted IL-10 in activated T cells ($P = 0.006$, Figure 3C), and also with TNF-α ($P = 0.0006$), IL-13 ($P = 0.0008$), IL-5 ($P = 0.048$), and IL-4 ($P = 0.012$) (data not shown), but all these correlations were independent of birth-order (Figure 3C).

The birth-order affects the general profile of activated cord blood T cells

In support of the conventional statistics, the PCA identified differences in overall surface marker and cytokine response levels between first-born and later-born neonates, respectively (Figure 4). Principal Component (PC) 1 was found to explain 27% of the variation in the data and to separate overall cytokines from surface markers (PC1, Figure 4B), but these patterns remained largely similar in first-born and later-born infants (PC1, Figure 4E). On the other hand, PC2, which explained 18% of the data variation, showed to separate T cell immunity in first-born and later-born infants ($P=0.02$, PC2, Figure 4E). Uniting the score

plot (Figure 4A) and loading plot (Figure 4B) for PC1 and PC2 again illustrated the correlation of IL-10 and $CD4^+CD25^+$ and their combined impact on the separation by maternal parity.

PC3, explaining 15% of the variation in data, appeared to cluster Th2 markers (CD30, CD278, IL-4, IL-5, IL-13), Treg (CD25, IL-10) and Th17 (IL-17), while separating them from Th1 markers (IFN-γ, TNF-α, CD154 and early activation (CD69)) (PC3, Figure 4C & D). PC3 did not relate to the birth-order (Figure 4E).

DISCUSSION

Main findings

We identified a functional divergence in activated cord blood T cells relating to birth-order. T cells from first-born children exhibited reduced IL-10 production and CD25 expression on CD4 helper T cells following activation. This weakened anti-inflammatory profile in higher birth-order newborns did not relate to the relative number of resting cord blood $CD4^+CD25^+$ T cells. Our findings imply that first-born newborns possess a T cell compartment that is programmed at birth to respond with lower IL-10 and CD25 expression in $CD4^+$ T cells upon stimulation as compared to second or later born children. Such changes could likely impact immune function and handling of environmental exposures during early life, and may as such influence later disease risk in first-born offspring.

Study strengths and limitations

It is a strength of this study that we used purified cord blood T cells allowing us to examine *in utero* imprinted properties of the T cell compartment without inference from other immune cells present in cord blood (23). Accordingly, we were able to document a clear *in utero* imprinting of the functional response from T cells by birth-order. To our knowledge this has not previously been demonstrated. We used anti-CD3/28-coated beads to polyclonally activate all subtypes of cord blood T cells. These beads mimic antigen-presenting cells and are believed to activate T cells in a physiological manner compared to more commonly used agents like *Staphylococcus* enterotoxin B, PMA/ionomycin and phytohemagglutinin (PHA). Addressing several T cell parameters such as T cell cytokines and surface activation marker expression allowed us, in a comprehensive manner, to identify specific functional entities affected by parity number. We tested for seven different

cytokines that promote immunity to intracellular pathogens (type-1; IFN-γ, TNF-α), extracellular pathogens (type-17; IL-17), helminthes (type-2; IL-4, IL-5, IL-13), as well as immune regulation (IL-10). These cytokine measures were combined with identification of the level of expression of seven surface markers on activated T cells (CD25, CD27, CD30, CD69, CD71, CD154, CD278) that are all enhanced during proliferation, but of which we have yet limited insight into in regard to their exact involvement in specific types of immune responses.

Based on our present finding pointing towards a reduced anti-inflammatory function in first-born newborns, it is a limitation that we did not perform a detailed characterization of Tregs in the cord blood of these newborns. Although no human lineage specific marker of Treg exists (24), $CD25^{hi}FoxP3^{+}CD127^{lo}$ are generally accepted as markers of Tregs (24). As we did not look specifically for Treg identification in this study, we had only data available for CD25. We therefore used CD25 expression patterns in unstimulated cord blood T cells to make a proxy for the percentage of Tregs (identified as $CD4^{+}CD25^{+}$) in cord blood. CD25 is constitutively expressed on Tregs, whereas activated effector T cells up-regulate CD25 expression. Since we observed no activation of $CD8^{+}$ T cells in unstimulated cord blood, we used this observation to imply that the number of recently activated effector $CD4^{+}$ T cells (with $CD25^{+}$) would accordingly be low in the cord blood of these infants. This is in line with earlier studies that have identified only very few activated T cells in cord blood (25). Thus, we assumed that the $CD4^{+}CD25^{+}$ subset identified here represent cord blood Tregs. This is further supported by a comparable percentage of $CD4^{+}CD25^{+}$ Treg cells in a previous study (26).

Interpretation

Our current finding supports the assumption that T cell functional properties of the newborn immune response are programmed *in utero* (27–29), and that birth-order may influence these priming events (27,30). We demonstrate that first-born infants display a reduced anti-inflammatory profile in T cells at birth. This suggests that immune regulatory mechanisms inducing maternal-fetal tolerance in the mother during pregnancy affects immune function in the offspring. This notion is also supported by a previous study showing a correlation of anti-inflammatory, but not pro-inflammatory, cytokines between mother and child (19).

The pleiotropic cytokine IL-10 that was decreased in activated T cells from first-born neonates has significant immune-modulatory properties affecting a broad range of immune and non-immune cells (reviewed in (31,32)). IL-10 is well-known for its repression of pro-inflammatory responses and limitation of inflammation-induced tissue disruption. A recent study, suggests that IL-10 itself may be important for Treg homeostasis in pregnancy (19). This may explain our current finding of a general correlation between the percentage of $CD4^+CD25^+$ cells in cord blood T cells and the secretion of IL-10 from stimulated T cells, irrespective of parity.

Up-regulation of CD25 (IL2Rα) expression is necessary for proliferation and differentiation of naïve T cells into effector T cells upon antigenic activation by antigen-presenting cells. Enhanced CD25 expression levels on $CD4^+$ T cells, without concomitant increase in other markers, is indicative of improved activity of a specific subset of $CD4^+$ T cells with suppressive properties (reviewed in (24,33)). Indeed, Tregs can suppress activation of by-stander T cells by up-regulating CD25 expression to scavenge IL-2 important for expansion of pro-inflammatory T cells. We observed an increased CD25 expression on $CD4^+$ T cells and an enhanced IL-10 secretion into culture medium, and speculate that a specific Treg cell subset could be responsible for this phenotype. One such subset could be the induced IL-10-

producing Tr1 subset that is itself promoted by IL-10 (34). Although it remains to be demonstrated, we speculate that transfer of the anti-inflammatory pregnancy milieu from mother to fetus may promote induction of regulatory Tr1 cells in the offspring, and that this specific subset may increase by birth-order. However, based on our exploratory experimental setup, we did not specifically examine the expansion and function of certain Treg populations. It will be of great interest to delineate this in future studies, since elucidation of the basis of *in utero* regulatory mechanisms could be of central importance to understand the reduced disease risk in higher-birth-order infants. We also speculate that enhanced IL-10 secretion from activated T cells after birth could confer some protection against excessive tissue pathology after exposure to infectious microbes, as well as increasing the level of peripheral tolerance to self and non-self antigens (35). Such disease-protective mechanisms could play part in the reduced disease risk seen in higher-birth-order infants (1).

It is widely believed that the birth-order-effect on disease risk arises due to postnatal exposures associated with having older children in the household, conceptualized by the hygiene hypothesis (36). The possibility of effects related to *in utero* priming of immunity as a cause of previous carriage of older siblings has received less attention. In the current study, we show that *in utero* priming of a T cell functional bias occurs before postnatal exposure to older siblings/children. Whether the factors mediating this effect is related to enhanced maternal exposure to infections during pregnancy (due to a higher microbial burden carried by older siblings), as proposed by Karmaus et al. (30), by feto-maternal communication of immunosuppressive mechanisms as a cause of the increased number of Tregs specific for paternal antigens found in secondary pregnancies (18), or by yet unidentified mechanisms still remain to be demonstrated. Based on our current results, it will however, be of interest to increase the focus on elucidation of the underlying mechanism as well as its contribution to *in utero* immune priming in shaping the risk of birth-order-affected diseases.

In conclusion, our present finding of decreased IL-10 secretion and CD25 expression on $CD4^+$ helper T cells in first-born children demonstrates that programming of T cells takes place *in utero* and depends on the number of maternal pregnancies. This finding may partially explain the enhanced risk of developing immune-related diseases in first-born individuals.

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468 **TABLES & FIGURES**

469 **Table 1.** Characteristics of the study population.

	Study cohort	**COPSAC$_{2010}$ cohort**	**P-value**[a]
	n = 28	n = 618	
Sex (n(%))			
Male	17 (61%)	313 (51%)	
Female	11 (39%)	305 (49%)	0.338
Parity (n(%))			
Primi	14 (50%)	293 (47%)	
Multi	14 (50%)	325 (53%)	0.848
Maternal Atopy (n(%))			
Yes	18 (64%)	295 (48%)	
No	10 (36%)	323 (52%)	0.248
Paternal Atopy (n(%))			
Yes	10 (36%)	346 (56%)	
No	18 (64%)	272 (44%)	0.440

470 [a]Fisher's exact test

471 **Table 2.** Influence of prenatal factors on cytokine secretion in activated cord blood T cells of
472 healthy neonates.

	IFN-γ[a] (pg/mL)	IL-10[a] (pg/mL)	IL-4[b] (pg/mL)	IL-5[a] (pg/mL)	IL-13[a] (pg/mL)	IL-17[b] (pg/mL)	TNF-α[a] (pg/mL)
Sex							
Male (n = 17)	75.4	22.9	2.2	1.9	259	3.0	1010
Female (n = 11)	98.5	26.0	3.9	2.2	254	4.2	1266
P-value	0.905	0.395	0.430	0.667	0.605	0.611	0.314
Adj. P-value[c]	0.963	0.300	0.653	0.761	0.872	0.831	0.246
Parity							
Primi (n = 14)	100.6	18.0	1.9	1.5	207	2.9	1055
Multi (n = 14)	68.4	30.2	3.7	2.6	307	4.1	1167
P-value	0.482	**0.007[d]**	0.069	0.170	0.067	0.246	0.636
Adj. P-value[c]	0.405	**0.011**	0.105	0.198	0.170	0.378	0.746
Maternal Atopy							
Yes (n = 18)	81.2	26.0	3.3	2.3	297	3.5	1146
No (n = 10)	90.5	20.6	2.0	1.6	185	3.6	1048
P-value	0.638	0.573	0.524	0.670	0.068	0.832	0.679
Adj. P-value[c]	0.766	0.940	0.650	0.585	0.541	0.946	0.511
Paternal Atopy							
Yes (n = 10)	69.8	19.9	1.7	1.4	195	3.3	1090
No (n = 18)	92.7	26.5	3.5	2.4	291	3.6	1123
P-value	0.496	0.475	0.286	0.160	0.095	0.912	0.846
Adj. P-value[c]	0.634	0.720	0.306	0.197	0.377	0.668	0.797

473 [a]P-value (unadjusted) calculated using Unpaired Student's t-test,
474 [b]P-value (unadjusted) calculated using Mann-Whitney-Wilcoxon test,
475 [c]P-values adjusted for possible confounders (sex, parity, parental atopy) calculated using
476 General Linear Models,
477 [d]P-value = 0.05 after Bonferroni correction

Table 3. Influence of prenatal factors on percentages $CD4^+$, $CD4^+CD25^+$ and $CD8^+$, respectively; and surface marker expressions in activated cord blood T cells of healthy neonates.

	$CD4^+$ Un-stimulated (%)	**$CD4CD25^+$** (%)	**CD71** (MFI)	**CD69[a]** (MFI)	**CD25** (MFI)	**CD154[a]** (MFI)	**CD30** (MFI)	**CD278** (MFI)	**CD27** (MFI)	**$CD8^+$ Un-stimulated** (%)	**CD71** (MFI)	**CD69** (MFI)	**CD25** (MFI)	**CD154** (MFI)	**CD30[a]** (MFI)	**CD278** (MFI)	**CD27** (MFI)
Sex																	
Male (n = 17)	68.3	8.6	12297	10216	33935	1834	255	25712	36424	27.5	13295	3812	51571	766	100	15263	52694
Female (n = 11)	73.9	8.1	12419	10443	36209	1883	237	27646	39755	21.5	13114	4159	57727	792	133	17536	56009
P-value	0.063	0.594	0.894	0.817	0.227	1.000	0.670	0.382	0.134	**0.048**	0.881	0.445	**0.041**	0.881	0.926	0.204	0.213
Adj. P-value[c]	0.225	0.958	0.858	0.998	0.064	0.961	0.871	0.289	0.256	0.199	0.819	0.457	0.054	0.536	0.489	0.269	0.214
Parity																	
Primi (n = 14)	69.8	7.7	12505	9517	31864	1896	233	25143	38029	25.6	13463	3628	52057	814	105	15334	54293
Multi (n = 14)	71.1	9.2	12185	11093	37793	1809	263	27800	37436	24.7	12983	4268	55921	738	122	16979	53700
P-value	0.660	0.113	0.723	0.210	**0.0003[d]**	0.701	0.470	0.215	0.789	0.772	0.693	0.144	0.200	0.650	0.603	0.350	0.822
Adj. P-value[c]	0.301	0.275	0.880	0.201	**0.0005**	0.716	0.682	0.297	0.837	0.374	0.902	0.179	0.138	0.942	0.516	0.337	0.911
Maternal Atopy																	
Yes (n = 18)	68.6	9.1	11775	10320	35317	1839	265	16121	53478	27.1	12790	4021	53456	672	108	16121	53478
No (n = 10)	74.0	7.3	13371	10278	33950	1878	218	16220	54930	21.6	14005.7	3816	54950	963	123	16220	54930
P-value	0.078	0.074	0.081	0.981	0.480	0.944	0.272	0.540	0.090	0.078	0.337	0.660	0.640	0.088	0.944	0.957	0.597
Adj. P-value[c]	0.106	0.215	0.438	0.490	0.610	0.515	0.629	0.611	0.507	0.081	0.849	0.850	0.886	0.052	0.504	0.754	0.684
Paternal Atopy																	
Yes (n = 10)	70.8	8.0	13568	9691	34540	1721	224	25800	39730	25.4	14486	3638	54150	780	101	15280	55530
No (n = 18)	70.3	8.7	11665	10646	34989	1926	262	26844	36622	25.0	12523	4120	53900	774	120	16643	53144
P-value	0.862	0.505	**0.035**	0.555	0.817	0.724	0.384	0.645	0.172	0.921	0.115	0.296	0.938	0.975	0.555	0.460	0.383
Adj. P-value[c]	0.494	0.758	0.200	0.408	0.585	0.236	0.721	0.965	0.441	0.306	0.263	0.423	0.870	0.262	0.449	0.500	0.362

[a]P-value (unadjusted) calculated using Unpaired Student's t-test, [b]P-value (unadjusted) calculated using Mann-Whitney-Wilcoxon test, [c]P-values adjusted for possible confounders (sex, parity, parental atopy) calculated using General Linear Models, [d]P = 0.004 after Bonferroni correction

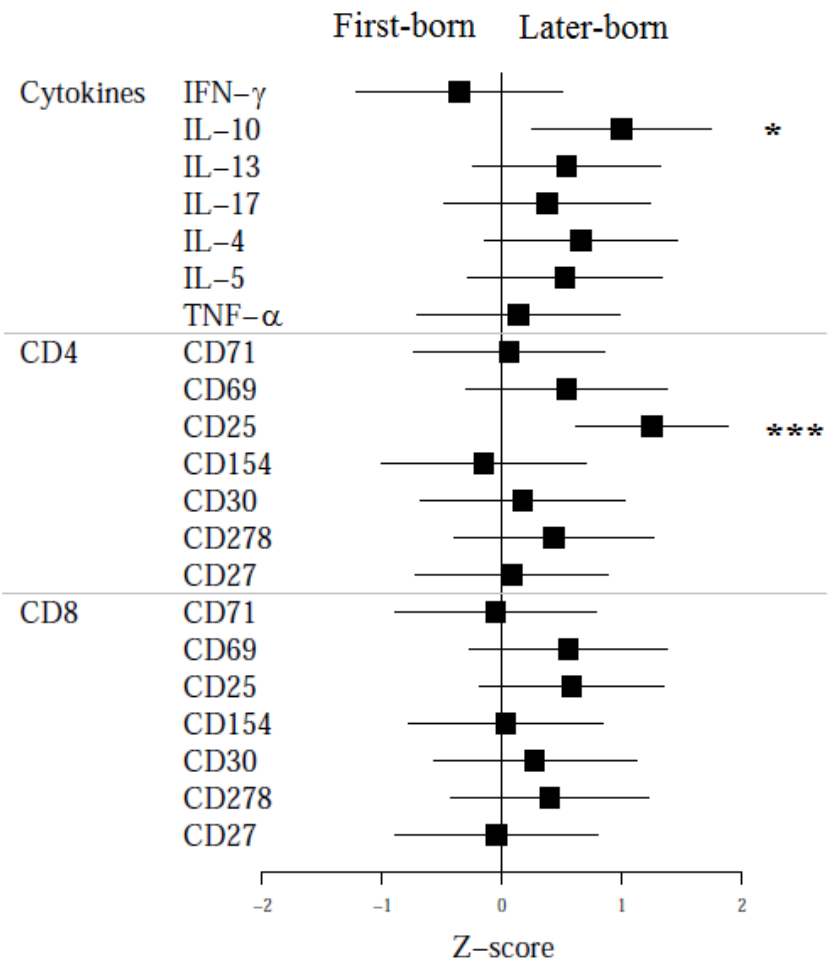
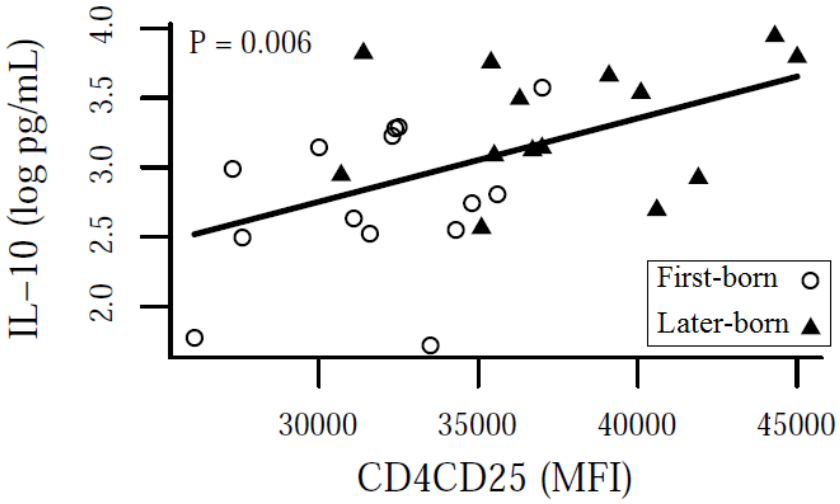


Figure 1. Differences in cytokine levels and surface marker expressions in activated cord blood T cells from healthy first-born infants versus later-born infants. Scaled, adjusted dependent variables (z-scores) expressed as differences between groups with corresponding 95% confidence interval. Statistical analysis by GLM with sex, parity and parental atopy as covariates. *, $P < 0.05$; ***, $P < 0.001$. CD154 (CD40L); CD278 (ICOS).



488

489 **Figure 2.** Correlation of CD25 expression on $CD4^+$ T cells and secreted IL-10 in activated
490 cord blood T cells of healthy first-born infants and later-born infants. IL-10 data are log-
491 transformed. Pearson's correlation analysis was used to calculate statistics.

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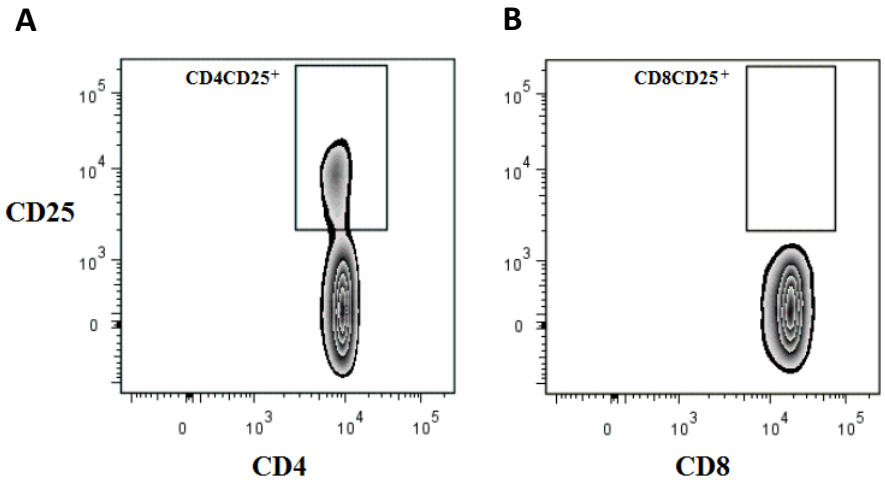

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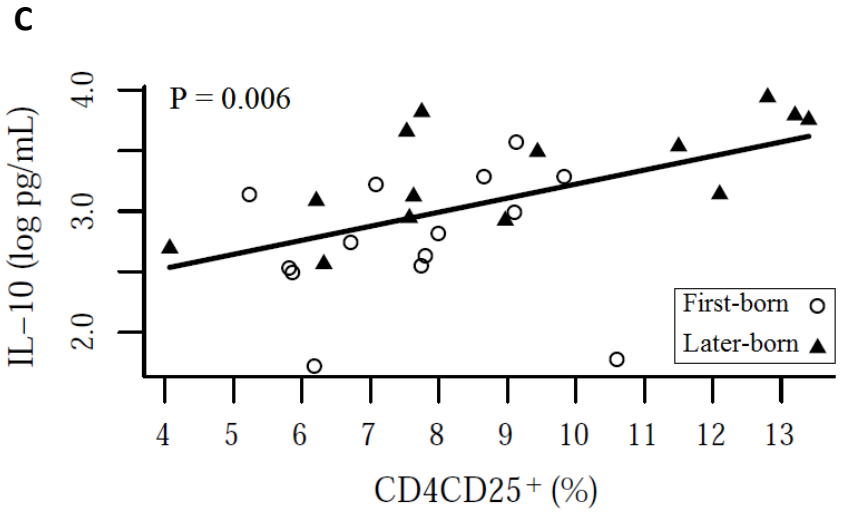

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495 **Figure 3.** Presence of $CD4^+CD25^+$ cells in un-stimulated cord blood T cells of healthy
496 neonates. **A.** Gating of $CD4^+CD25^+$ cells **B.** Gating of $CD8^+CD25^+$ cells. **A-B.** Cord blood T
497 cells were isolated as described in METHODS. One representative analysis out of 28 is
498 shown. **C.** Correlation of the percentage of $CD4^+CD25^+$ in un-stimulated cord blood T cells
499 and secreted IL-10 in activated cord blood T cells of healthy first-born infants and later-born

infants. IL-10 levels are log-transformed. Pearson's correlation analysis was used to calculate statistics.

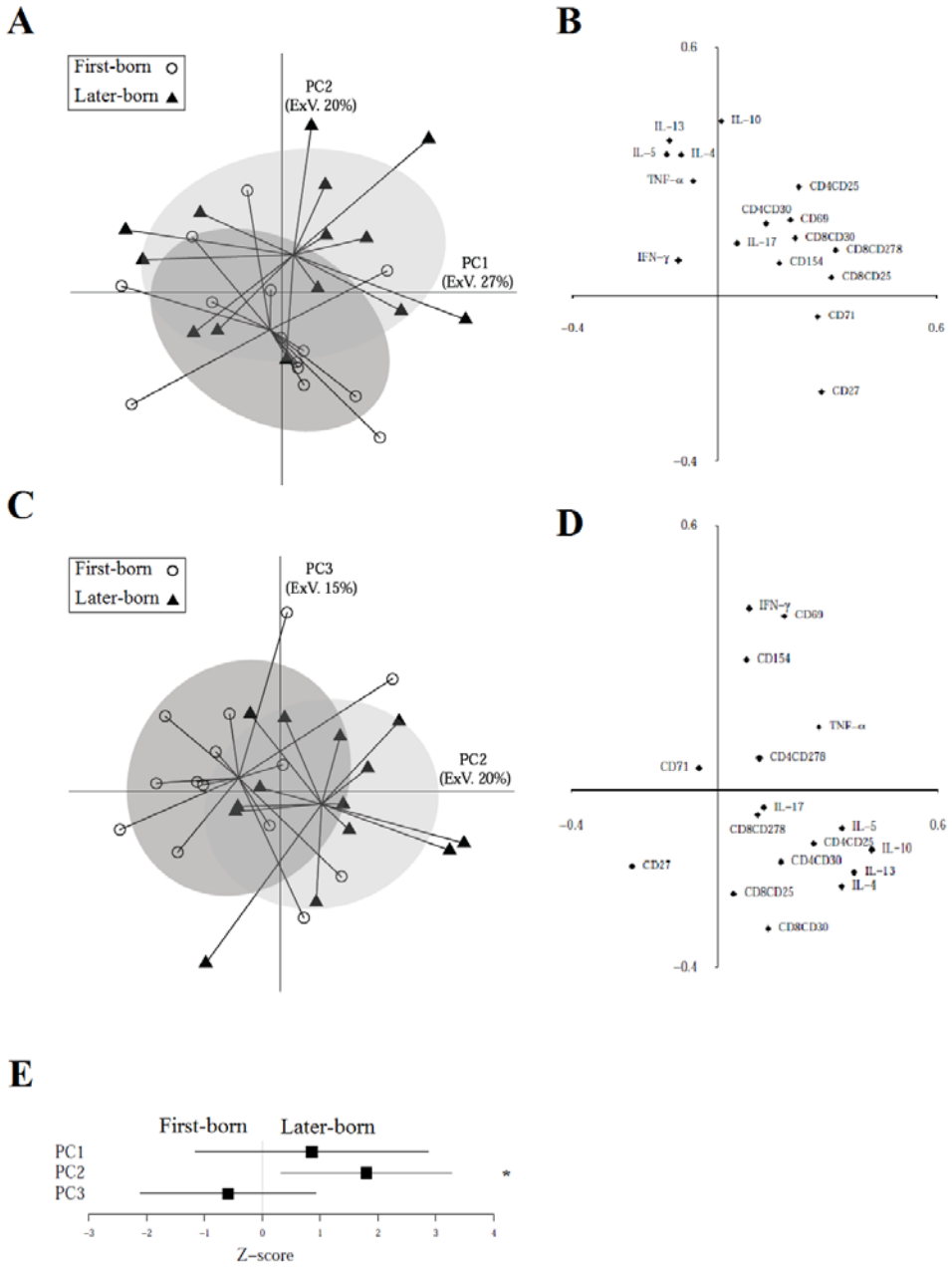


Figure 4. Principal component analysis of the combined immune profiles of activated cord blood T cells across first-born and later-born neonates. **A, C.** Score-plots grouped by birth-order. Principal components (PCs) are plotted using the ade4 package in R with each

newborn represented by a symbol. The mean of each birth-order group is the center of the ellipse and the colored area covers 67% of the samples belonging to this group. **B, D.** Loading-plots illustrate the systematic distribution of immune mediators determined by PCs. In cases where expression of a given activation marker were similar on CD4 and CD8 cells, only the marker name is indicated. **E.** Statistics on PCs divided by parity. Data are scaled, adjusted PCs (z-scores) based on a GLM with sex, parity and parental atopy as covariates; and expressed as differences between groups with corresponding 95% confidence interval. *, $P < 0.05$.

514 **SUPPLEMENTARY**

515 **Table S1. Data on sex of baby; maternal and paternal atopy by parity status**
516

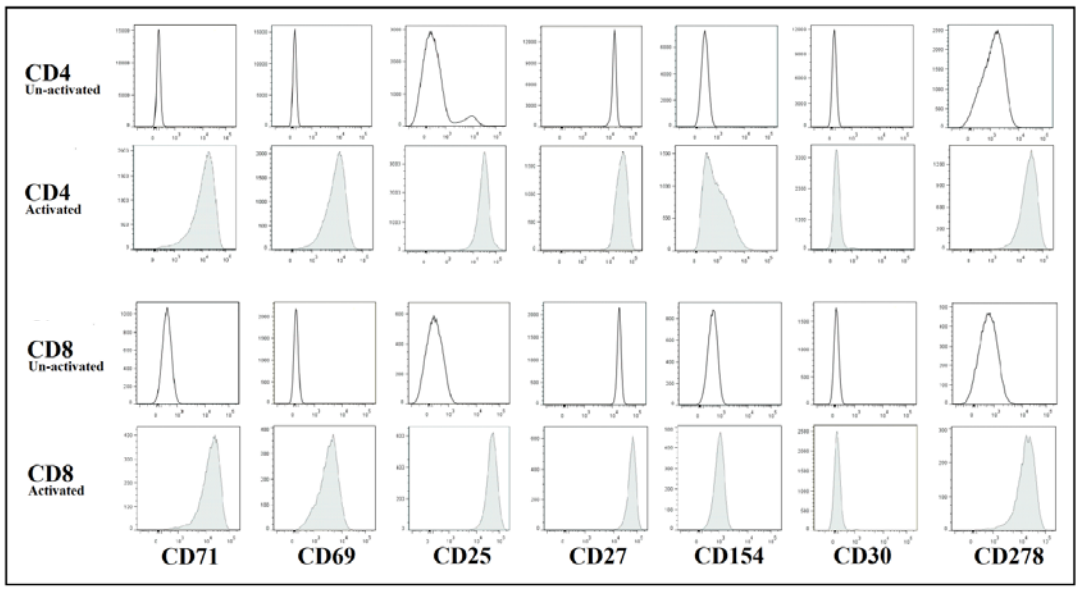
Parity	Sex	Maternal atopy	Paternal atopy
Primi (n=14)	6 females	1	3
	8 males	6	3
Multi (n=14)	5 females	4	1
	9 males	7	3

517

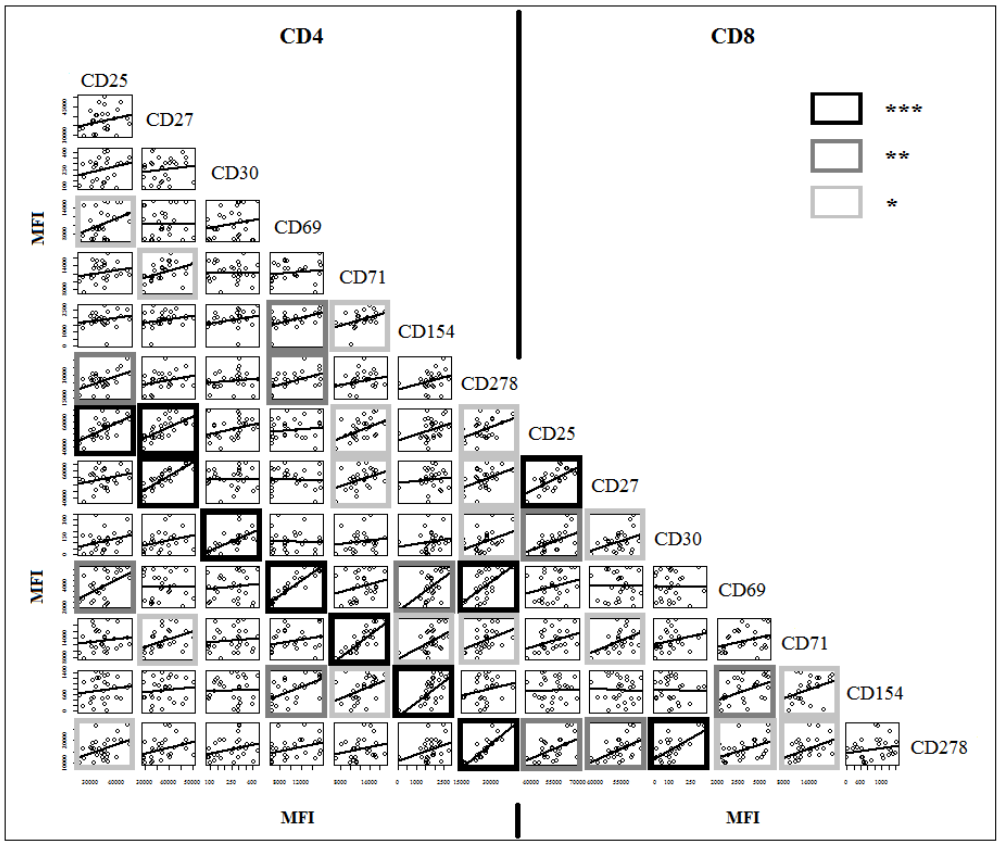
518

Figure S1. Activation propensity of the neonatal T cell compartment.

To explore the activation propensity of isolated neonatal $CD4^+$ and $CD8^+$ T cells at birth, we profiled the polyclonal response to anti-CD3/CD28 using multicolor flow cytometry. Our profile included early activation markers CD69, CD71, CD25 (aIL2-R) as well as CD27, CD30, CD154 (CD40L) and CD278 (ICOS). Upon polyclonal stimulation, we measured a complete activation of both $CD4^+$ and $CD8^+$ T cells with 1.6 – 4000 fold changes in surface marker expression level dependent on the marker. Data shows representative histograms of surface marker expression levels before and after polyclonal activation of cord blood $CD4^+$ and $CD8^+$ T cells for 24h.



532 **Figure S2. Correlation matrix of surface marker expression levels in activated cord blood**
533 **CD4$^+$ and CD8$^+$ T cells of healthy neonates.** Data show regression lines and Pearson
534 correlation statistics: *, P < 0.05, **, P < 0.01; ***P < 0.001.



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536

Figure S3. Relatedness of activation markers and effector cytokines expressed in activated cord blood T cells. Data show correlation matrix of cytokine and CD4+ surface marker expression levels in activated cord blood T cells of healthy neonates. Shown are regression lines and Pearson correlation statistics: *, $P < 0.05$, **, $P < 0.01$; ***$P < 0.001$.

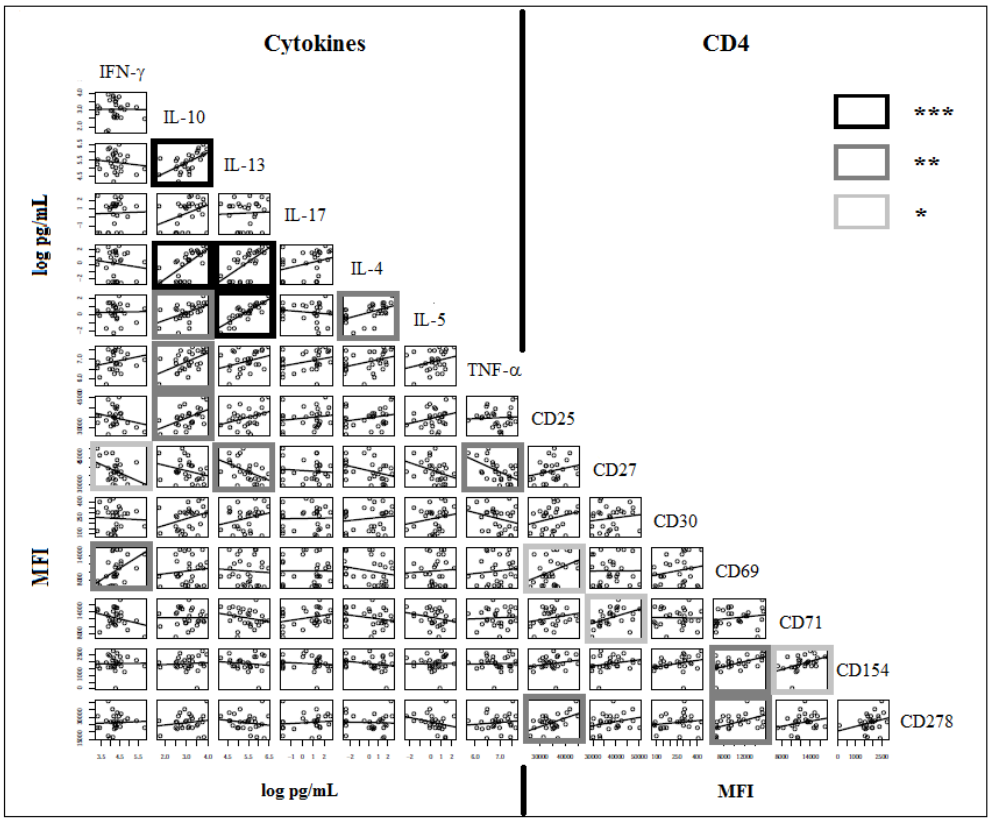

Figure S4. Correlation matrix of cytokine and CD8$^+$ surface marker expression levels in activated cord blood T cells of healthy neonates. Data display regression lines and Pearson correlation statistics: *, P < 0.05, **, P < 0.01; ***P < 0.001.